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RESEARCH ARTICLE

Dexmedetomidine mitigates isoflurane-induced neurodegeneration in fetal rats during the second trimester of pregnancy

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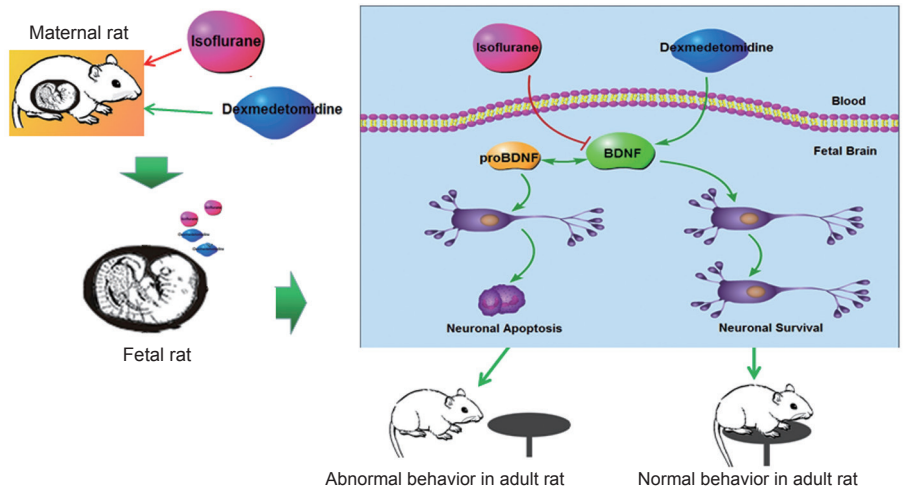
Graphical Abstract

Dexmedetomidine can reduce neurodegeneration induced by isoflurane in fetal rats during the second trimester of pregnancy



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Abstract

Dexmedetomidine has significant neuroprotective effects. However, whether its protective effects can reduce neurotoxicity caused by isoflurane in fetal brain during the second trimester of pregnancy remains unclear. In this study, timed-pregnancy rats at gestational day 14 spontaneously inhaled 1.5% isoflurane for 4 hours, and were intraperitoneally injected with dexmedetomidine at dosages of 5, 10, 20, and 20 μg/kg 15 minutes before inhalation and after inhalation for 2 hours. Our results demonstrate that 4 hours after inhaling isoflurane, 20 μg/kg dexmedetomidine visibly mitigated isoflurane-induced neuronal apoptosis, reversed downregulation of brain-derived neurotrophic factor expression, and lessened decreased spatial learning and memory ability in adulthood in the fetal rats. Altogether, these findings indicate that dexmedetomidine can reduce neurodegeneration induced by isoflurane in fetal rats during the second trimester of pregnancy. Further, brain-derived neurotrophic factor participates in this process.

Key Words: *nerve regeneration; dexmedetomidine; isoflurane; fetal rat; apoptosis; brain-derived neurotrophic factor; behavior; neuroprotection; neurodegeneration; neural regeneration*

Introduction

Non-obstetric maternal and fetal surgery during pregnancy is increasing with the development of operational and anesthesia techniques (Ní Mhuireachtaigh and O'Gorman, 2006; Mellon et al., 2007). These types of operation are usually performed in the second trimester of pregnancy (Tran, 2010; Upadya and Saneesh, 2016; Elbabaa et al., 2017), a time when the fetal brain reaches a peak for neural development, synapse occurrence, and neuronal migration (Zhao et al., 2011). During this period, even slight changes in the environment may alter developmental processes. High concentrations of inhaled anesthetics, including isoflurane (Iso), are commonly

used in maternal and fetal surgeries to inhibit uterine contraction and reduce the risk of premature labor or miscarriage (Yoo et al., 2006; Van De Velde and De Buck, 2007). However, there is now substantial evidence supporting the neurotoxic effects of general anesthetic agents on the immature brain (Wang et al., 2009; Li et al., 2013; Broad et al., 2016; Olutoye et al., 2016; Wang et al., 2017). Based on this, a growing number of studies are focused on reducing such neurological damage. Various drugs, such as Xenon (Ma et al., 2007), dexmedetomidine (Dex) (Sanders et al., 2009; Li et al., 2016; Alam et al., 2017), lithium ion (Straiko et al., 2009; Zanni et al., 2017), melatonin (Yon et al., 2006; Miyamoto, 2011), and astragaloside (Sun et al., 2016) are being tested as protective agents. Currently, most studies use newborn rats, and studies on fetal rats during the second trimester of pregnancy have rarely been reported. Because Dex is widely used in clinical treatment (Afonso and Reis, 2012), we hypothesized that combined use of Dex and Iso would result in decreased Iso-induced nerve injury in fetal rats. Thus, to investigate the protective effect of Dex on Iso-induced nerve injury in fetal rats and explore its mechanism, we designed our study to simulate clinical anesthesia during non-obstetric operations of the second trimester of pregnancy by administering long-term inhalation of Iso to rats at gestational day 14.

Materials and Methods

In order to investigate the short-term and long-term protective effect of Dex, the study was divided into two parts, including laboratory and behavioral testing.

Part I

Animals

A total of 70 female specific-pathogen-free Sprague-Dawley rats (aged 40–60 days, weighing 200–300 g before pregnancy), with no external genital organ defects and no excessive excitement or other abnormal behavior were provided by Guangdong Medical Laboratory Animal Center in China (No. SCXK-Yue-2013-0002). Rats were excluded if they: had respiratory and heart rates that decreased > 50% during inhalation, severe hypoxia or low blood glucose levels (as shown by blood gas analysis), and < 3 fetuses upon hysterectomy delivery. The experiment was performed in timed-pregnancy, inseminated female rats and their developing fetuses, with approval by the Animal Ethics Committee of The Third Affiliated Hospital of Guangzhou Medical University in China (approval No. 2017052).

Experimental treatment of maternal rats

Female rats were fed in polypropylene cages ($60 \times 40 \times 20$ cm^3) with 12-hour dark-light cycles and constant temperature and humidity. Animals were allowed free access to food and water. Female rats were mated with males for one night and maternal vaginal smears examined the next day. Females were considered pregnant if sperm was observed. The day after mating was recorded as gestational day 1. All pregnant rats were fed alone in the same environment until gestational day 14.

In total, 70 pregnant rats were randomized into 7 groups at gestational day 14 (n = 10): Dex5 + Iso, Dex10 + Iso, Dex20 + Iso, normal saline (NS) + Iso, Dex20 + yohimbine (Yoh) + Iso, Dex20 + oxygen (Oxy), and control.

Drug injection: In the Dex5 + Iso, Dex10 + Iso, Dex20 + Iso, and Dex20 + Oxy groups, Dex (2 μg/mL; Jiansu Hengrui Medicine Co., Ltd., Lianyungang, Jiangsu Province, China; license No. 13090632) was intraperitoneally injected into maternal rats at dosages of 5, 10, 20, and 20 μg/kg, respectively, 15 minutes before Iso inhalation and 2 hours after Iso or Oxy inhalation. Rats in the Dex20 + Yoh + Iso group were administered Dex at 20 μg/kg and Yoh (50 μg/mL; Sigma-Aldrich, St. Louis, MO, USA; license No. Y3125) at 500 μg/kg at the same time points and same injection site. Rats in the NS + Iso group were injected with 10 mL/kg normal saline. Rats in the control group did not receive any treatment. The dosages of Dex were in accordance with a previous study (Sanders et al., 2009). Moreover, 5 μg/kg in rats is equivalent to 1 μg/kg in humans, while 10 μg/kg and 20 μg/kg are also commonly used doses in animal studies. In pre-experiments, pregnant rats at the second trimester of pregnancy tolerated these three doses well, but severe bradypnea and hypotension sometimes occurred if the Dex dose was > 25 μg/kg when combined with Iso inhalation. Thus, 20 μg/kg was set as the maximum Dex dose in this study.

Gas inhalation: Gas inhalation was performed in a plexiglass box ($20 \times 12 \times 12$ cm^3). The concentration of Iso was monitored *via* a catheter placed in the box. Pregnant rats in the Dex5 + Iso, Dex10 + Iso, Dex20 + Iso, NS + Iso, and Dex20 + Yoh + Iso groups spontaneously inhaled a mixture of 1.5% Iso (Abbott Laboratories, Abbott Park, IL, USA; license No. 8670828) and 98.5% Oxy for 4 hours to expose fetal rats to Iso. Rats in the Dex20 + Oxy group inhaled 100% Oxy for 4 hours in the same box. Rats in the control group were not given any treatment. The ambient temperature was kept at 32 ± 1°C using a heating lamp or ice pack. Respiratory and heart rates were monitored before intraperitoneal drug injection and after gas inhalation for 2 and 4 hours. Tail artery blood samples were collected after gas inhalation for 4 hours, and blood gas analysis performed. Further, pH, partial pressure of oxygen (PO_2), partial pressure of carbon dioxide (PCO_2), and glucose values were recorded. At the end of gas inhalation, all rats were exposed to 100% Oxy for recovery, and returned to their cages after resumption of normal activities. Hysterectomy delivery for pregnant rats was performed 4 hours later and fetus counts recorded.

Experimental treatment of fetal rats

Hysterectomy delivery was performed in maternal rats after intraperitoneal injection of 3% chloral hydrate at 10 mL/kg. Two fetal rats were randomly selected for each maternal rat. Fetal rats were isolated on ice and fixed in 4% paraformaldehyde.

Sampling

Fetal rat brain tissue was fixed in 4% paraformaldehyde for 24–36 hours. For paraffin sections, fixed tissue was cut coronally 2 mm from the front of fetal rat brains. Tissue was dehydrated sequentially in 70%, 80%, 90%, and 95% ethanol for 60 minutes each, followed by 100% ethanol twice for 1 hour each, and then 50% xylene–50% ethanol once and

100% xylene twice for 15 minutes each. Samples were immersed twice in paraffin wax (56–58°C), for 60 minutes each time. Tissue was then embedded into paraffin blocks and sliced into two consecutive sections for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and immunohistochemistry assay.

TUNEL assay

In accordance with a TUNEL assay kit (Promega, Madison, WI, USA), tissue sections were deparaffinized twice (5 minutes each), immersed sequentially through graded ethanol washes (100%, 95%, 85%, 75%, and 50%) and pure water for 5 minutes each wash, and then immersed in 0.85% NaCl for 5 minutes. After washing in PBS for 5 minutes, sections were fixed in 4% paraformaldehyde for 15 minutes, and washed twice with PBS for 5 minutes each wash. Proteinase K (20 μg/mL) was added and sections incubated at room temperature for 30 minutes. After two PBS washes (5 minutes each), sections were fixed in 4% paraformaldehyde for 10 minutes, and washed twice with PBS for 5 minutes each wash. Sections were equilibrated with equilibration buffer at room temperature for 10 minutes, and then rTdT incubation buffer was added and incubated at 37°C for 60 minutes. After washing with 2 × standard saline citrate for 15 minutes, sections were immersed twice in PBS for 5 minutes each time. Sections were then blocked in 3% H_2O_2 at room temperature for 10 minutes, washed twice with PBS (5 minutes each wash), treated with streptavidin horseradish peroxidase (1:500 diluted in PBS) at room temperature for 30 minutes, and again washed twice with PBS (5 minutes each). After visualization with 3,3′-diaminobenzidine, sections were washed three times with PBS (5 minutes each wash), counterstained with hematoxylin, immersed in differentiation liquid for several seconds, and then washed with water for bluing. Sections were dehydrated through a graded alcohol series (50%, 75%, 85%, 95%, and 100%) for 10 minutes each, permeabilized twice with xylene for 10 minutes each, and mounted for microscopic examination. Data were collected blindly. Three non-overlapping 400-fold microscope fields were randomly selected to calculate apoptotic cell percentage (number of TUNEL-positive cells/total cell number × 100%).

Immunohistochemical assay

Sections were deparaffinized twice in xylene for 10 minutes each time, and dehydrated through a graded alcohol series (100%, 95%, 85%, 75%, and 50%) and pure water for 5 minutes each. Sections were washed in PBS for 5 minutes, incubated in 3% H_2O_2 at room temperature in a moist box for 10 minutes, and then washed three times in PBS for 5 minutes each wash. Next, antigen retrieval was performed in a microwave oven with citric acid buffer. Specifically, sections were heated in a microwave oven on medium-high power until boiling and then continued at low power for 8 minutes. After cooling down to room temperature, slides were immersed in PBS for 5 minutes, incubated with 10% normal goat serum in a moist box for 30 minutes with primary antibody (rabbit monoclonal anti-brain-derived neurotrophic factor [BDNF]; 1/200; Abcam, Cambridge, Bristol, UK) at 4°C overnight, and then immersed three times in PBS for 5 minutes each time. Afterwards, samples were incubated with secondary antibody (goat anti-rabbit, 1:1; Envision TM Detection Kit, Peroxidase/DAB, DAKO, Glostrup, DENMARK) in a moist box at 26°C for 30 minutes, washed three times with PBS for 5 minutes each, and visualized with 3,3′-diaminobenzidine. Next, sections were washed three times with PBS for 5 minutes each, counterstained with hematoxylin, immersed in differentiation liquid for several seconds, and then washed with water for bluing. Slides were dehydrated through a graded alcohol series (50%, 75%, 85%, 95%, and 100%) for 10 minutes each, and permeabilized twice with xylene for 10 minutes each. Data were collected blindly. Three non-overlapping 400-fold fields were randomly selected using a microscope (Olympus, Tokyo, Japan). The mean optical density method was used to calculate mean optical density values. Mean values are presented.

Part II

Animals

A total of 50 female specific-pathogen-free Sprague-Dawley rats (aged 45–60 days, weighing 200–300 g before pregnancy) were provided by Guangdong Medical Laboratory Animal Center in China (No. SCXK-Yue-2013-0002). The experiment was performed in timed-pregnancy, inseminated female rats and their mature offspring. The selection criteria were the same as Part I.

Experimental treatment of maternal rats

Female rats were subjected to the same conditions as in Part I, until gestational day 14. To evaluate long-term effectiveness of protection by Dex, 50 pregnant rats were randomized into 5 groups (n = 10): Dex20 + Iso, NS + Iso, Dex20 + Yoh + Iso, Dex20 + Oxy, and controls.

Drug injection: In the Dex20 + Iso and Dex20 + Oxy groups, Dex (2 μg/mL) was intraperitoneally injected into maternal rats at a dosage of 20 μg/kg, 15 minutes before inhalation and 2 hours after inhalation. Rats in the Dex20 + Yoh + Iso group were administered Dex at 20 μg/kg and Yoh (50 μg/mL) at 500 μg/kg at the same time points and same injection site. Rats in the NS + Iso group were injected with 10 mL/kg normal saline. Animals in the control group did not receive any treatment.

Gas inhalation: Pregnant rats in the Dex20 + Iso, NS + Iso, and Dex20 + Yoh + Iso groups spontaneously inhaled a mixture of 1.5% Iso and 98.5% Oxy for 4 hours to expose fetal rats to Iso. Rats in the Dex20 + Oxy group inhaled 100% Oxy for 4 hours in the same box. Rats in the control group were not given any treatment. At the end of gas inhalation, all rats were exposed to 100% Oxy for recovery, and returned to their cages after resumption of normal activities. All pregnant rats were fed until natural delivery. Pup counts were recorded.

Behavioral testing

Offspring were allowed to mature until postnatal day 56. To avoid the influence of gender, two male pups were randomly selected from each litter for Morris water maze testing. The

Table 1 Short-term protective effect of dexmedetomidine (Dex) on body weight of maternal rats on gestational day 14 and fetal count in isoflurane (Iso)-inhaled maternal rats during the second trimester of pregnancy

Group	Weight (g)	Fetus count
Dex5+Iso	251.000±12.884	10.700±1.889
Dex10+Iso	253.600±12.851	9.800±2.821
Dex20+Iso	252.400±13.243	11.200±2.700
NS+Iso	259.500±16.928	11.500±2.415
Dex20+Yoh+Iso	262.200±13.113	11.700±2.541
Dex20+Oxy	256.400±13.451	11.300±2.406
Control	254.100±16.928	11.300±3.093
F	0.879	0.616
P	0.516	0.716

Data are expressed as the mean ± SD, with 10 maternal rats in each group. One-way analysis of variance followed by the least significant difference test was performed. NS: Normal saline; Yoh: yohimbine; Oxy: oxygen; Dex5, 10, 20: Dex was intraperitoneally injected at dosages of 5, 10, and 20 μg/kg.

Table 3 Long-time protective effect of dexmedetomidine (Dex) on body weight of maternal rats on gestational day 14 and pups on day 56, and pup count in isoflurane (Iso)-inhaled maternal rats during the second trimester of pregnancy

Group	Weight of maternal rats (g)	Weight of pups (g)	Pup count
Dex20+Iso	249.900±12.503	292.050±19.533	10.800±2.348
NS+Iso	254.600±14.668	296.900±15.556	9.700±2.830
Dex20+Yoh+Iso	252.700±14.221	289.400±19.386	11.500±1.958
Dex20+Oxy	258.300±13.639	290.000±17.120	11.300±2.869
Control	251.500±10.533	297.350±16.936	11.200±2.741
F	0.600	0.450	0.778
P	0.664	0.772	0.545

Data are expressed as the mean ± SD, with 10 maternal rats in each group. One-way analysis of variance followed by the least significant difference test was performed. NS: Normal saline; Yoh: yohimbine; Oxy: oxygen; Dex20: Dex was intraperitoneally injected at dosage of 20 μg/kg.

Table 2 Short-term protective effect of dexmedetomidine (Dex) on blood gas analysis results of isoflurane (Iso)-inhaled maternal rats during the second trimester of pregnancy

Group	pH	PO_2 (mmHg)	PCO_2 (mmHg)	Glucose (mg/dL)
Dex5+Iso	7.467±0.064	122.600±5.317*	29.150±4.136	132.300±21.945
Dex10+Iso	7.489±0.058	120.500±7.517*	29.870±5.288	130.900±20.083
Dex20+Iso	7.474±0.083	122.200±10.369*	29.890±4.897	132.100±20.798
NS+Iso	7.469±0.048	119.900±9.195*	31.990±2.855	127.600±17.513
Dex20+Yoh+Iso	7.461±0.079	122.300±6.325*	32.730±4.322	131.300±16.166
Dex20+Oxy	7.455±0.089	121.700±11.265*	29.960±4.694	126.700±17.914
Control	7.486±0.057	86.600±5.338	31.600±4.247	135.800±19.078
F	0.319	25.989	0.934	0.258
P	0.925	0.000	0.477	0.954

Data are expressed as mean ± SD, with 10 maternal rats in each group. One-way analysis of variance followed by the least significant difference test was performed. *P < 0.05, *vs.* control group. NS: Normal saline; Yoh: yohimbine; Oxy: oxygen; PCO_2: partial pressure of carbon dioxide; PO_2: partial pressure of oxygen; Dex5, 10, 20: Dex was intraperitoneally injected at dosages of 5, 10, and 20 μg/kg.

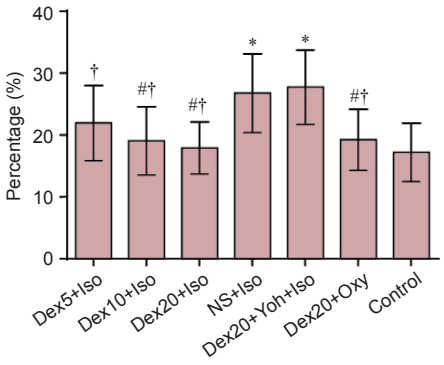


Figure 1 Short-term protective effect of dexmedetomidine (Dex) on cell apoptotis in fetal brain following isoflurane (Iso)-induced neurodegeneration during the second trimester of pregnancy.
Data are expressed as mean ± SD, with 10 maternal rats in each group. One-way analysis of variance followed by the least significant difference test was performed. *P < 0.05, *vs.* control group; #P < 0.05, *vs.* NS + Iso group; †P < 0.05, *vs.* Dex20 + Yoh + Iso group. NS: Normal saline; Yoh: yohimbine; Oxy: oxygen; Dex 5, 10, 20: Dex was intraperitoneally injected at dosages of 5, 10, 20 μg/ng.

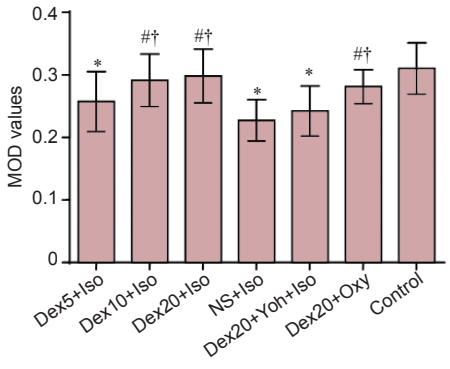
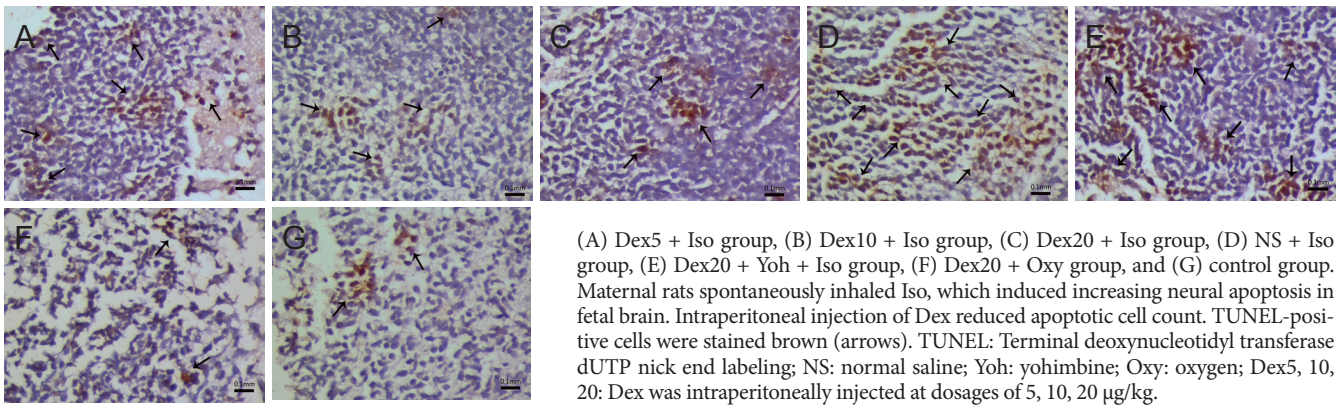


Figure 2 Short-term protective effect of dexmedetomidine (Dex) on brain-derived neurotrophic factor (BDNF) immunoreactivity in fetal brain following isoflurane (Iso)-induced neurodegeneration during the second trimester of pregnancy.
Data are expressed as mean ± SD, with 10 maternal rats in each group. One-way analysis of variance followed by the least significant difference test was performed. *P < 0.05, *vs.* control group; #P < 0.05, *vs.* NS + Iso group; †P < 0.05, *vs.* Dex20 + Yoh + Iso group. NS: Normal saline; Yoh: yohimbine; Oxy: oxygen; Dex5, 10, 20: Dex was intraperitoneally injected at dosages of 5, 10, 20 μg/ng.

Table 4 Long-term protective effect of dexmedetomidine (Dex) on escape latency in Morris water maze from day 1 to day 4 in isoflurane (Iso)-inhaled maternal rats during the second trimester of pregnancy

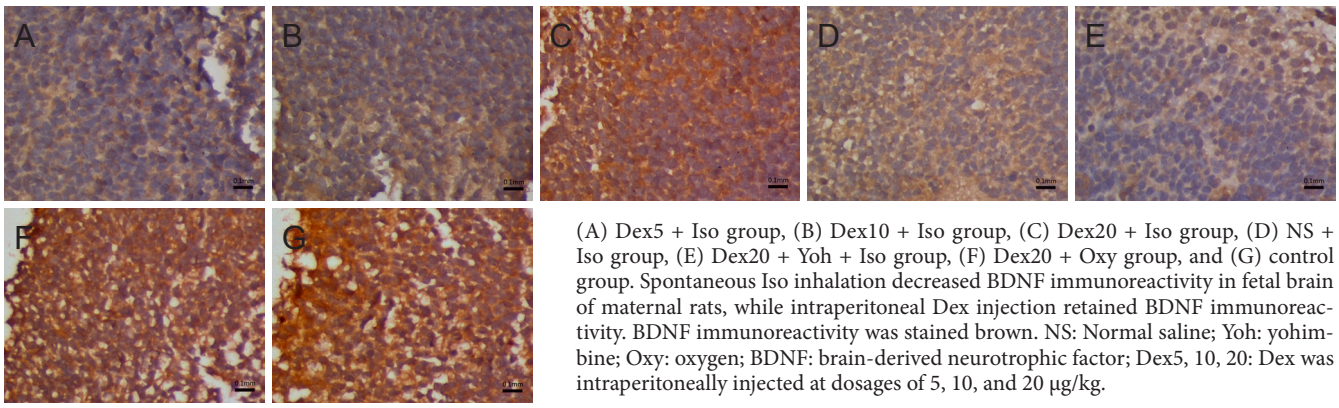
Group	Day 1	Day 2	Day 3	Day 4	Sum	*F*	*P*
Dex20+Iso	100.500±19.080	90.050±20.893	66.450±22.688	44.150±17.662$^{\#\dagger}$	75.288±29.377	33.171	0.000
NS+Iso	98.900±21.126	89.200±17.377	86.850±17.995*	69.950±21.526*	86.225±21.583	5.903	0.003
Dex20+Yoh+Iso	100.900±17.220	88.600±18.539	81.250±12.148*	65.200±17.150*	83.988±20.516	16.268	0.000
Dex20+Oxy	105.500±15.089	86.800±16.506	64.200±20.947$^{\#}$	47.300±20.481$^{\#\dagger}$	75.950±28.521	25.501	0.000
Control	103.650±17.953	92.700±12.555	66.150±19.512	45.000±17.756$^{\#}$	76.875±28.450	28.785	0.000
Sum	101.890±17.608	89.470±16.781	72.980±20.465	54.320±21.314	79.665±26.107	99.348^{a}	0.000^{a}
F	0.211	0.155	2.957	4.173	1.437^{a}	2.591^{b}	
P	0.931	0.960	0.030	0.006	0.237^{a}	0.004^{b}	

Data are expressed as the mean ± SD, with 10 maternal rats in each group. Repeated measures two-way analysis of variance was used. ^{a}Main effect, ^{b}crossover effect. *P < 0.05, *vs.* control group; #P < 0.05, *vs.* NS + Iso group; †P < 0.05, *vs.* Dex20 + Yoh + Iso group. NS: Normal saline; Yoh: yohimbine; Oxy: oxygen; Dex20: Dex was intraperitoneally injected at dosage of 20 μg/kg.



(A) Dex5 + Iso group, (B) Dex10 + Iso group, (C) Dex20 + Iso group, (D) NS + Iso group, (E) Dex20 + Yoh + Iso group, (F) Dex20 + Oxy group, and (G) control group. Maternal rats spontaneously inhaled Iso, which induced increasing neural apoptosis in fetal brain. Intraperitoneal injection of Dex reduced apoptotic cell count. TUNEL-positive cells were stained brown (arrows). TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling; NS: normal saline; Yoh: yohimbine; Oxy: oxygen; Dex5, 10, 20: Dex was intraperitoneally injected at dosages of 5, 10, 20 μg/kg.

Figure 3 Short-term protective effect of dexmedetomidine (Dex) on cell apoptosis in fetal brain following isoflurane (Iso)-induced neurodegeneration during the second trimester of pregnancy (TUNEL staining, × 400).



(A) Dex5 + Iso group, (B) Dex10 + Iso group, (C) Dex20 + Iso group, (D) NS + Iso group, (E) Dex20 + Yoh + Iso group, (F) Dex20 + Oxy group, and (G) control group. Spontaneous Iso inhalation decreased BDNF immunoreactivity in fetal brain of maternal rats, while intraperitoneal Dex injection retained BDNF immunoreactivity. BDNF immunoreactivity was stained brown. NS: Normal saline; Yoh: yohimbine; Oxy: oxygen; BDNF: brain-derived neurotrophic factor; Dex5, 10, 20: Dex was intraperitoneally injected at dosages of 5, 10, and 20 μg/kg.

Figure 4 Short-term protective effect of dexmedetomidine (Dex) on BDNF immunoreactivity in fetal brain following isoflurane (Iso)-induced neurodegeneration during the second trimester of pregnancy (immunohistochemical staining, × 400).

weight of these pups was recorded. The Morris water maze was provided by Chendu Taimeng Technology Ltd., China. All testing sessions were performed between 9 a.m. to 5 p.m. in the same room, which was homogenously illuminated by normal fluorescent room light at 50 lux. The Morris water maze was performed in a pool with a black internal coating (120 cm diameter, 60 cm altitude) that was filled with water (25°C). Movement was tracked by a camera placed 2.5 m overhead. Spatial cues were present around the pool, and the pool was divided into four quadrants (northwest, northeast, southeast, and southwest). The test was divided into two stages for spatial learning and spatial memory. An escape platform (10 cm diameter) was placed in the northeast quadrant (2 cm submerged, 30 cm from edge). Pups were trained to find the location of the platform in four acquisition trials (maximal swimming time 120 seconds; 30 seconds on the platform; inter-trial interval 60 minutes) per day during four consecutive days. Pups were led to the platform if they did not find it within 120 seconds. Starting positions during the four trial days were: southeast,

Table 5 Long-term protective effect of dexmedetomidine (Dex) on time of first platform crossing and frequency of platform crossings in isoflurane (Iso)-inhaled maternal rats during the second trimester of pregnancy

Group	Time of first crossing (second)	Frequency (times/120 seconds)
Dex20+Iso	30.500±13.844$^{\#\dagger}$	2.500±1.000$^{\#\dagger}$
NS+Iso	48.500±22.914*	1.550±0.550*
Dex20+Yoh+Iso	46.900±15.560*	1.650±0.530*
Dex20+Oxy	30.050±14.488$^{\#\dagger}$	2.400±0.775$^{\#\dagger}$
Control	31.850±14.648	2.600±0.907
F	3.124	4.146
P	0.024	0.006

Data are expressed as the mean ± SD, with 10 maternal rats in each group. One-way analysis of variance followed by the least significant difference test was performed. *$P < 0.05$, *vs.* control group; #$P < 0.05$, *vs.* NS + Iso group; †$P < 0.05$, *vs.* Dex20 + Yoh + Iso group. NS: Normal saline; Yoh: yohimbine; Oxy: oxygen; Dex20: Dex was intraperitoneally injected at dosage of 20 μg/kg.

southwest, northwest, and northeast. Escape latency to find the hidden platform from the southeast start position was scored. Escape latency represents spatial learning ability. After 120-seconds of swimming, the offspring were placed back in their home cage dried by towel to avoid hyposthenia and hypothermia and allowed to eat freely. On day 5, all offspring performed a single probe trial with the platform removed, starting from the southeast position. Offspring were allowed to swim for 120 seconds. The time of first platform crossing (at former platform location) and frequency of platform crossings, which represent spatial memory ability, were recorded.

Statistical analysis

Measurement data are presented as the mean ± SD. SPSS17.0 statistical software (SPSS, Chicago, IL, USA) was used for statistical analysis and GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA) was used for graph plotting. To reduce sampling errors, the method used was described by a previous study (Wang et al., 2009). Two fetal siblings or two pups were randomly selected for each test and mean values collected for statistical analysis. Accordingly, the number of samples for each group was still 10. Data for latency time to find the hidden platform from day 1 to day 4 in the Morris water maze were analyzed by repeated measures two-way analysis of variance. One-way analysis of variance was used to compare different treatment groups, and the least significant difference test for paired comparisons of the other data. Differences with $P < 0.05$ were considered to show statistical significance.

Results

Part I

General information

Body weight was similar and not statistically different in maternal rats in each group on gestational day 14 ($F = 0.525$, $P = 0.717$). Fetus count was also similar ($F = 0.552$, $P = 0.699$) (**Table 1**).

Blood gas analysis

Values for pH, PCO_2, and glucose were similar and not statistically different in each group ($F = 0.598$, $P = 0.666$; $F = 0.552$, $P = 0.699$; and $F = 0.274$, $P = 0.894$, respectively). However, PO_2 levels showed significant differences ($F = 25.989$, $P < 0.001$). Specifically, PO_2 was significantly lower in the control group compared with the other groups ($P < 0.05$) (**Table 2**). This is because animals in the control group were not supplied with 100% Oxy.

Apoptotic cells

Apoptotic cell percentage showed significant group variations ($F = 6.145$, $P < 0.001$). Compared with the control group, no significant differences were found in the Dex10 + Iso, Dex20 + Iso, and Dex20 + Oxy groups ($P > 0.05$). However, apoptotic cell percentage was slightly increased in the Dex5 + Iso group ($P > 0.05$), and significantly increased in the NS + Iso and Dex20 + Yoh + Iso groups ($P < 0.05$). Compared with the NS + Iso group, the percentage was significantly decreased in the Dex10 + Iso, Dex20 + Iso, and Dex20 + Oxy groups ($P < 0.05$). The percentage in the Dex5 + Iso group was slightly decreased ($P > 0.05$), and similar in the Dex20 + Yoh + Iso and NS + Iso groups ($P > 0.05$). Percentages in the Dex5 + Iso, Dex10 + Iso, and Dex20 + Iso groups were significantly lower compared with the Dex20 + Yoh + Iso group ($P < 0.05$) (**Figures 1**, **3**).

BDNF immunoreactivity

BDNF immunoreactivity showed significant group variations ($F = 6.015$, $P < 0.001$). BDNF immunoreactivity was similar in the Dex10 + Iso, Dex20 + Iso, and Dex20 + Oxy, and control groups ($P > 0.05$). BDNF immunoreactivity was significantly decreased in the Dex5 + Iso, NS + Iso, and Dex20 + Yoh + Iso groups compared with the control group ($P < 0.05$). Compared with the NS + Iso group, BDNF immunoreactivity was significantly increased in the Dex10 + Iso, Dex20 + Iso, and Dex20 + Oxy groups ($P < 0.05$), and similar in the Dex5 + Iso, Dex20 + Yoh + Iso, and NS + Iso groups ($P > 0.05$). BDNF immunoreactivity was significantly decreased in the Dex10 + Iso, Dex20 + Iso, and Dex20 + Oxy groups compared with the Dex20 + Yoh + Iso group ($P < 0.05$). The Dex5 + Iso group showed similar immunoreactivity as the Dex20 + Yoh + Iso group ($P > 0.05$; **Figures 2**, **4**).

Part II

General information

Body weight was similar in maternal rats on gestational day 14 and pups on day 56 in each group ($F = 0.600$, $P = 0.664$; $F = 0.450$, $P = 0.772$). Pup count was also similar ($F = 0.778$, $P = 0.545$; **Table 3**).

Behavioral changes

In the Morris water maze, escape latency was measured from day 1 to day 4. It was significantly different on different days ($F = 99.348$, $P < 0.001$) and was shortest on day 4 in all groups. On day 3, there were no significant differences in escape latency between the groups ($P > 0.05$). Escape latency

was similar between the Dex20 + Yoh + Iso and NS + Iso groups ($P > 0.05$), which was significantly increased compared with the control group ($P < 0.05$). Escape latency was reduced in the Dex20 + Oxy group compared with the NS + Iso group ($P < 0.05$), which was similar to the control group ($P > 0.05$). On day 4, the Dex20 + Iso, Dex20 + Oxy, and control groups showed approximately similar escape latencies ($P > 0.05$), which were all significantly less than the NS + Iso group ($P < 0.05$). The Dex20 + Iso group showed a significantly reduced escape latency compared with the Dex20 + Yoh + Iso group ($P < 0.05$; **Table 4**).

On day 5, data for time of first platform crossing and frequency of platform crossings in the hidden platform search test showed significant group differences ($F = 3.124$, $P = 0.024$; $F = 4.146$, $P = 0.006$). Time of first platform crossing in the Dex20 + Iso, Dex20 + Oxy, and control groups were similar ($P > 0.05$), but significantly less than the NS + Iso group ($P < 0.05$). Further, it was significantly longer in the Dex20 + Yoh + Iso group compared with the control group ($P < 0.05$), yet similar to the NS + Iso group ($P > 0.05$). The Dex20 + Iso group showed a significantly shorter time than the Dex20 + Yoh + Iso group ($P < 0.05$). Frequency of platform crossings was similar in the Dex20 + Iso, Dex20 + Oxy, and control groups ($P > 0.05$), but significantly more in the NS + Iso group ($P < 0.05$). The Dex20 + Yoh + Iso group showed significantly less than the control group ($P < 0.05$) and NS + Iso group. The Dex20 + Iso group showed significantly more than the Dex20 + Yoh + Iso group ($P < 0.05$; **Table 5**).

Discussion

Dex is a highly efficient and selective α2-adrenoceptor (α2-AR) agonist with a distribution half-life of approximately 6 minutes and elimination half-time of approximately 2 hours (Ebert et al., 2000). Dex can pass through the placenta and affect the fetus. The rate of placental transfer 2 hours after administration is 12.5 ± 5.1% in humans (Ala-Kokko et al., 1997). Dex has a prominent neuroprotective effect (Ma et al., 2005; Rodríguez-González et al., 2016; Wang et al., 2016; Kim et al., 2017), and was the first drug discovered to exert a protective effect on Iso-induced neurocognitive dysfunction (Sanders et al., 2009). Yoh is a highly selective α2-AR inhibitor that competitively antagonizes Dex binding to α2-AR. Further, Yoh penetrates the blood-brain barrier to reverse the effects of Dex on the central nervous system (Ren et al., 2016).

A dose of 1.5% Iso is close to 1 minimum alveolar concentration (MAC) in rats (Mazze et al., 1985), and can lead to significant neurodegeneration in immature rat brain (Palanisamy et al., 2011; Su et al., 2015). The results of our study suggest that neurodegeneration occurs in fetal rat brain at gestational day 14 when maternal rats inhale 1.5% Iso for 4 hours. Moreover, the effect was associated short-term with increasing abnormal apoptosis, and long-term with decreasing spatial learning and memory ability. Nevertheless, apoptosis decreased significantly in the groups receiving Dex at 10 and 20 μg/kg, suggesting that Dex effectively inhibits Iso-induced abnormal neuronal apoptosis in fetal brain. The results of the Dex5 + Iso group lie between the NS + Iso group and control group, with no statistically significant differences compared with either group. This suggests that the action of Dex is dose-dependent, and 5 μg/kg Dex may not be sufficient to reduce abnormal apoptosis. The maximum dose of Dex used in this study was 20 μg/kg, but apoptosis increased in the Dex20 + Yoh + Iso group. This suggests that the neuroprotective effect of Dex can be blocked by Yoh and that the neuroprotective effect on immature brain at gestational day 14 is mainly caused by Dex. In the Morris water maze, Iso exposure at the fetal stage significantly inhibited spatial learning and memory ability in adult offspring. This result is consistent with previous reports (Li et al., 2007; Palanisamy et al., 2011). Combined use with Dex can protect behavioral ability, suggesting that the protective effects of Dex are long-term. Nevertheless, the results of our study also show that the protective effect may be simultaneously attenuated by Yoh, further confirming that the neuroprotective effect is induced by Dex.

Although many reports suggest that the NMDA/GABA mechanism plays a key role in the toxic effects of general anesthetics on the immature brain of perinatal rats (Ben-Ari, 2002; Hansen et al., 2004; Xiang et al., 2008; Stratmann, 2011), an alternative mechanism (*e.g.*, neurotrophic mechanism *via* BDNF) may be more important in brain damage and protection of fetal rats. This is because fetal brain structure in the second trimester is not necessarily the same as in the perinatal period, including NMDA receptor deficiency and immature GABA receptor function (Sanders et al., 2009; Palanisamy et al., 2011).

BDNF plays important roles (Liao et al., 2014), and can promote neuronal survival, regulate synaptic development and plasticity of the central nervous system, and modulate neurotransmitter release. Moreover, it is an important target for general anesthetic drugs (Yuan et al., 2017). Inhibited BDNF expression is observed during injury (Lemkuil et al., 2011). Brain expression levels of BDNF are affected by both Iso and Dex (Degos et al., 2013). Pro-BDNF is the immature form of BDNF in the brain, and the balance between BDNF and pro-BDNF is critical for cell survival (Li et al., 2017). Iso downregulates BDNF expression by inhibiting pro-BDNF conversion to BDNF (Lemkuil et al., 2011), and thus reducing BDNF binding to its receptor, Trk (Lv et al., 2016). In turn, this leads to reduced activation of Akt and ERK1/2 phosphorylation (Lu et al., 2006; Li et al., 2014; Zhang et al., 2015; Lv et al., 2017). Thus, neuronal activity and apoptosis are increased with abnormal excitement (Sanders et al., 2010; Zhang et al., 2014). Simultaneously, increased pro-BDNF activates another receptor, p75NTR (Head et al., 2009), and downstream RhoA-associated protein kinase by acting on RhoA (Bito et al., 2000). This leads to actin depolymerization and growth cone collapse as well as loss of immature dendritic spines and synapses (Chang et al., 2016). Cytoskeletal reorganization also occurs resulting in nerve cell apoptosis (Coleman and Olson, 2002; Dubreuil et al., 2003). However, Dex acts on the α2-AR in astrocytes and increases transcription of Bdnf4 and Bdnf5 fragments, and BDNF secretion. This lead to increased amounts of brain BDNF (Hertz et al., 2010; Reynolds et al., 2015) and reduces the degree of nerve

injury induced by Iso (Ryu et al., 2014; Stary et al., 2015). Anti-BDNF antibody inhibits BDNF expression regulated by Dex, and abolishes the protective effect of Dex on excitability and perinatal rat brain (Lemkuil et al., 2011). This also indicates that upregulation of BDNF expression is an important mechanism for Dex to improve neuronal survival. Here, BDNF expression levels in all groups were determined after treatment with Iso and Dex alone, or in combination. Our results show that Iso exposure significantly suppresses BDNF expression in fetal rat brain, which is nearly restored to normal levels when Dex is added at 10 μg/kg or 20 μg/kg. Dex failed to restore BDNF levels in the Dex20 + Yoh + Iso group, suggesting that Yoh abolishes the neuroprotective effect of Dex. Dex alone had only a marginal effect on BDNF expression, but reversed its downregulation by Iso. Meanwhile, our findings also show that increased BDNF expression is coincident with reduced cerebral neuronal apoptosis in fetal rats at gestational day 14, indicating that upregulation of BDNF expression may be an important reason for the neuroprotective effect of Dex.

Safety of Dex application during pregnancy has been controversial (Tariq et al., 2008). Studies have found that Dex can increase spontaneous contractions and contraction frequency of isolated rats in the uterus during the first and second trimester, therefore application of Dex in the second trimester increases the risk of miscarriage (Öcal et al., 2013). Due to its vasoconstrictive effect, Dex may also cause uteroplacental vasoconstriction and induce hypoxia ischemia in fetal rat. However, in our study, Dex application either alone or in combination to pregnant rats did not induce miscarriage. Indeed, the degree of neuronal apoptosis in fetal rats of the Dex20 + Oxy group was similar to that in the control group. Therefore, we infer that Dex at a dosage of no more than 20 μg/kg does not induce miscarriage and has little effect on uteroplacental vasoconstriction.

As other methods and drugs to protect the immature brain from injury induced by anesthetics are limited for various reasons, Dex has been widely used in clinical practice. It shows protective effects on neurons of fetal rats and does not interfere with normal apoptosis when applied alone, suggesting that its action is safe, effective, and worthy of further investigation.

Application of Dex can reduce neurodegeneration in intrauterine fetal rats treated by Iso, and this effect can be antagonized by Yoh. Dex restores BDNF expression that is downregulated by Iso in fetal rat brain and that may participate in neuroprotective effects of Dex.

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